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Immunoreactive FMRFamide in the Nervous System of the Earthworm, *Eisenia foetida*

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ABSTRACT—A number of large and small cells immunoreactive to FMRFamide (Phe-Met-Arg-Phe-NH_2) antiserum were found in the cerebral, subesophageal and segmental ganglia, and in the ganglia of the pharyngeal plexus of the earthworm, *Eisenia foetida*. The cerebral ganglion sends the immunoreactive fibers to the subesophageal ganglion through the pharyngeal connectives. The subesophageal ganglion projects the fibers to the segmental ganglia. From the cerabral and subesophageal ganglia, the nerve bundles containing immunoreactive nerve fibers proceed anteriorly towards the prostomium and the ventral region of the anterior segments, respectively. From the ganglia in the pharyngeal plexus, the immunoreactive fibers proceed towards the pharynx. From each segmental ganglion, three immunoreactive segmental nerve trunks emerge bilaterally and extend to the body wall. Then they run dorsally between the circular and longitudinal muscle layers of the body wall. Immunoreactive fibers were observed between the circular muscle layer and the epithelial cell layer of the skin. Furthermore, immunoreactive fibers were observed between the epithelial cell layer and the circular muscle layer of the alimentary canal. Immunoreactive products are occasionally present in contact with the blood vessels in the cerebral and subesophageal ganglia. FMRFamide seems to act both as a neurotransmitter or neuromodulator and as a neurohormone in the earthworm.

INTRODUCTION

Since Scharrer [1] first described neurosecretory cells in the central nervous system of the earthworm, many investigators have studied neurosecretion in the earthworm by cytological and physiological techniques. It has been suggested that some types of neurosecretory cell in the cerebral ganglion are involved in the growth and regeneration of nervous centers, in the reproduction of the earthworm [2], and in the regulation of the osmotic properties of the coelomic fluid in the earthworm [3, 4]. Biochemically, the occurrence of serotonin, dopamine, noradrenaline and octopamine has been established in the earthworm's central nervous system [5–8]. However, recent investigations in the earthworm have tended to concentrate on the search for biologically active peptides in the nervous system by predominantly immunocytochemical techniques, which were developed primarily for vertebrate peptide hormones. For example, the following peptides have been found immunohistochemically in the earthworm's nervous system: pancreatic polypeptide (PP) and vasoactive intestinal peptide (VIP) [9], α-endorphin [10], β-endorphin [11], adrenocorticotropic hormone (ACTH) [12], substance P (SP)[12, 13], Leu-enkephalin [13, 14], Met-enkephalin, human growth-hormone-releasing factor (h-GHRF), dynorphin [13], gastrin/cholecystokinin (CCK) [13–15], corticotropin-releasing factor (CRF) [13, 16], vasopressin (AVP) and oxytocin (OXT) [17]. Thus, the nervous system of the earthworm contains many biological active peptides previously identified in vertebrates.

FMRFamide (Phe-Met-Arg-Phe-NH_2) and its related peptides, wich were first described in the clam *Macrocallista nimbosa* by Price and Greenberg [18, 19], have been detected chemically [20] and immunohistochemically not only in the molluscan nervous system [21–25], but also in the

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nervous system of other species of invertebrates and vertebrates [25–27]. Thus, FMRFamide and its related peptides are widely distributed in invertebrates and vertebrates, but these compounds have not yet been examined in the earthworm.

This report describes the existence and distribution of FMRFamide-like immunoreactivtiy in the nervous system of the earthworm, *Eisenia foetida.*

MATERIALS AND METHODS

Adult earthworms, *Eisenia foetida,* were collected on the laboratory campus in May. The animals were cut into 4 or 5 pieces and fixed in Bouin's solution without acetic acid for 2-3 hr at room temperature. After fixation, the pieces were dehydrated through an ethanol series, cleared in xylene, and embedded in paraffin. Serial frontal, sagittal and horizontal sections were cut at 6 μm thickness. Deparaffinized sections were immunostained by the peroxidase-antiperoxidase (PAP) method of Sternberger *et al.* [28]. To block the endogenous peroxidase activity, hydrated sections were soaked in 3% H_2O_2 in distilled water for 15 min and rinsed for 15 min in 0.01 M phosphate-buffered saline (PBS; pH 7.2). They were preincubated in 1% normal goat serum (Polysciences Inc., Warrington, Pennsylvania) in PBS for 30 min to remove background staining. The sections were then incubated with FMRFamide antiserum (1:4000; Immuno Nuclear Corp., Stillwater, Minnesota) overnight at 4°C. Subsequently, the sections were washed in PBS for 15 min and incubated with goat anti-rabbit IgG (1:200; Polysci-

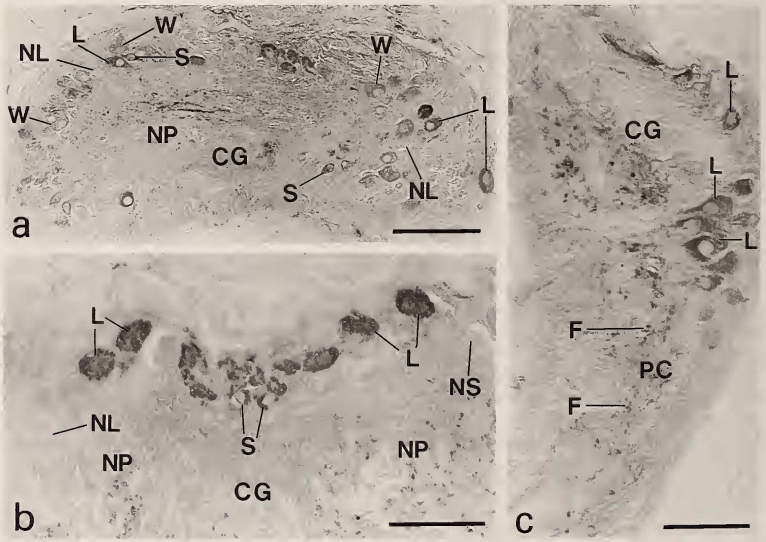

Fig. 1. a. Cross section of the earthworm, *Eisenia foetida,* through the dorso-posterior portion of the cerebral ganglion (CG), showing FMRFamide-like immunoreactive cells. b. Cross section through the anterior dorso-medial region of CG, showing a group of small cells (S) with FMRFamide-like immunoreactivity. A few large cells (L) are present on the both sides of the group. c. Cross section showing large cells (L) with FMRFamide-like immunoreactivity in the region of the CG, from which the pharyngeal connective (PC) emerges. F: immunoreactive fiber bundle, NL: non-immunoreactive large cells, NP: neuropile, W: weakly immunoreactive cells. Scale, a: 100 μm, b: 50 μm, c: 50 μm.

ences Inc.) for 60 min. After the sections were washed in PBS for 15 min, they were incubated in PAP complex (1:100; Dako Corp., Copenhagen) for 30 min. Each incubation was performed in a moist chamber at room temperature. For the peroxidase reaction, slides were immersed in 0.02% 3, 3′-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemicals, Osaka) in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.005% H_2O_2, for about 1–3 min, and then slides were washed in PBS and distilled water for 5 min each. The sections were dehydrated through an ethanol series, cleared in xylens, and mounted.

The specificity of the immunoreaction was checked for the various sections of the cerebral and subesophageal ganglia, the epidermal layer of the skin and the alimentary canal, by the preabsorption test using the antiserum incubated for 24 hr with synthetic FMRFamide (5 μg/ml; Peptide Institute, Osaka, Japan).

RESULTS

The sections treated with FMRFamide antiserum preabsorbed with the antigen (FMRFamide) did not show any evidence of immunoreactivity in any tissues examined.

Cerebral ganglion

In the dorso-posterior portion of the cerebral ganglion, many large cells (approximately 20 μm in diameter) and small cells (approximately 10 μm in

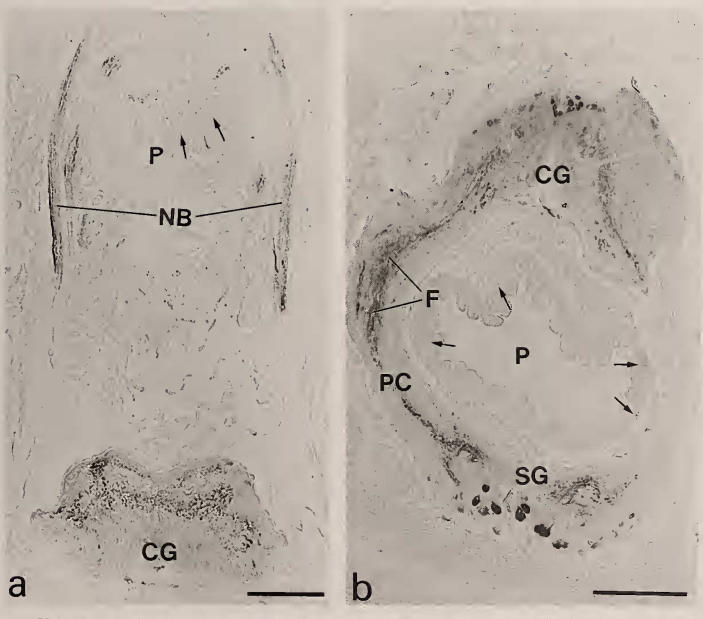

Fig. 2. a. Horizontal section showing FMRFamide-like immunoreactive nerve bundles (NB) proceeding anteriorly from the cerebral ganglion (CG) to the prostomium. b. Cross section showing the FMRFamide-like immunoreactive fibers (F) in the pharyngeal connective (PC). Arrows show immunoreactive products between the epithelial and circular muscle layers of the pharynx (P). SG: subesophageal ganglion. Scale, a: 150 μm, b: 200 μm.

diameter) immunoreacted strongly or weakly to FMRFamide antiserum (Fig. 1a). These cells were located predominantly near the surface of the cerebral ganglion. Among these immunoreactive cells, non-immunoreactive large and small cells of similar sizes were always present (Fig. 1a, b). Between the immunoreactive cells and the surface of the cerebral ganglion, many small non-immunoreactive cells (aproximately 10 μm in diameter) were present. Few immunoreactive large cells were also found in the anterior region, but none in the ventral region of the cerebral ganglion. In the dorso-anterior region, a few small immunoreactive cells (approximately 10 μm in diameter) were gathered in a group in the median portion (Fig. 1b) and a few large immunoreactive cells (approximately 15 μm in diameter) were distributed near both sides of the group (Fig. 1b). In addition, several large immunoreactive cells were found bilaterally in the regions where the pharyngeal connectives emerged (Fig. 1c).

The nerve fibers of these immunoreactive cells proceeded first to the neuropile which was located in the middle portion of the cerebral ganglion (Fig. 1a). Two immunoreactive nerve bundles proceeded anteriorly from the ventral side of the cerebral ganglion to the prostomium (Fig. 2a). Other fibers proceeded to the subesophageal ganglion along the pharyngeal connectives (Figs. 1c, 2b). Immunoreactive products were occasionally present in contact with the blood vessels in the

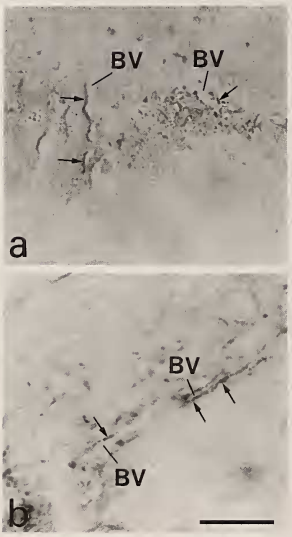

Fig. 3. a. Cross section of the cerebral ganglion. b. Cross section of the subesophageal ganglion. Arrows show FMRFamide-like immunoreactive products in close contact with blood vessels (BV). Scale, 50 μm.

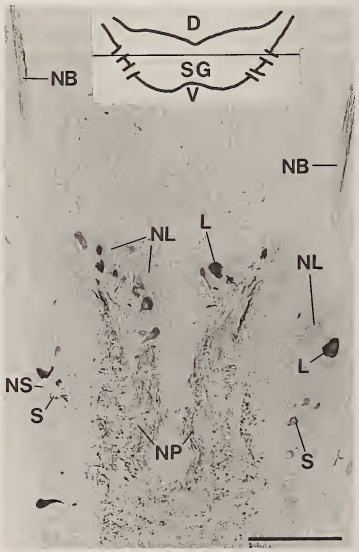

Fig. 4. Horizontal section of the subesophageal ganglion (SG) at the level shown in the inset. Immunoreactive and non-immunoreactive large and small cells are found. D: dorsal, L: immunoreactive large cells, NB: two nerve bundles containing immunoreactive nerve fibers which proceed towards the anterior segments. Two other bundles emerging from each side of the posterior region of SG are not shown in this figure. NL: non-immunoreactive large cells, NP: neuropile, NS: non-immunoreactive small cells, S: immunoreactive small cells, V: ventral. Scale, 100 μm.

neuropile of the cerebral ganglion (Fig. 3a).

Subesophageal ganglion

Many large nerve cells (approximately 15 μm in diameter) and small cells (approximately 10 μm in diameter) and their fibers in the subesophageal ganglion immunoreacted with FMRFamide antiserum (Figs. 2b, 4). The processes of the immunoreactive cells entered the neuropile in the middle portion of the ganglion and then proceeded posteriorly to join the ventral nerve cord (Fig. 4). Non-immunoreactive cells were also found in the ganglion (Fig. 4). From both sides of the anterior and posterior regions of the subesophageal ganglion, two thick immunoreactive nerve bundles emerged, respectively, and proceeded anteriorly towards the ventral region of the anterior segments (Fig. 4). Immunoreactive products were often present in close contact with blood vessels (Fig. 3b).

Pharyngeal plexus

FMRFamide-like immunoreactive cells (approximately 10 μm in diameter) were present in the ganglia of the pharyngeal plexus (Fig. 5a). Nerve tracts connecting these ganglia with the pharyngeal connectives were observed. The tracts contained many immunoreactive fibers (Fig. 5b). Furthermore, it was observed that immunoreactive fibers proceeded anteriorly or posteriorly from the ganglia towards the pharynx (Fig. 5b).

Segmental ganglion and skin

FMRFamide-like immunoreactivity was observed in many large and small cells located bilaterally and ventrally in the ganglion of each segment. Figures 6a and 6b show the cross and horizontal sections, respectively. of the anterior sixth segment. From these cells, strong immunoreactive fibers proceeded to the neuropiles located on both sides of the central portion of the ventral nerve cord (Fig. 6a). Figure 7 shows the horizontal section cut through the neuropile shown in Figure 6a. The immunoreactive cells were observed in the peripheral region and two wide longitudinal immunoreactive nerve tracts were observed (Fig. 7). However, many non-immunoreactive cells were also found in the segmental ganglia (Fig. 7). The immunoreactive cell numbers in the segmental ganglia of the posterior region of the body were smaller than those in the anterior and the middle regions.

From each segmental ganglion, three nerve tracts containing immunoreactive fibers emerged

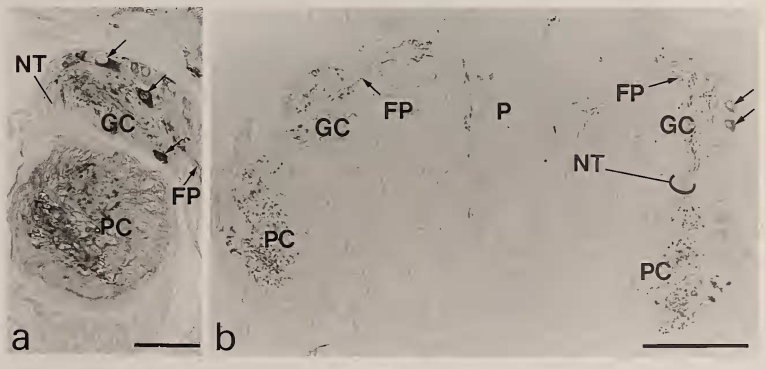

Fig. 5. Horizontal section of the pharyngeal plexus. a. Immunoreactive cells (arrows) in a ganglion (GC) of the pharyngeal plexus. b. Section showing the topographic relations between GC, pharyngeal connective (PC) and pharynx (P). Arrows show immunoreactive cells. FP. immunoreactive fiber bundles proceeding to the pharynx. NT: nerve tract containing immunoreactive fibers between GC and PC. Scale, a: 50 μm, b: 100 μm.

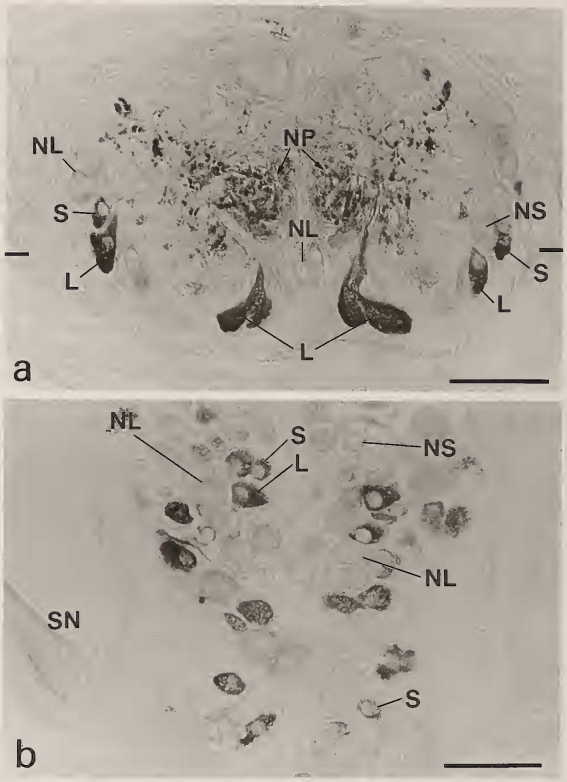

Fig. 6. a. Cross section of the 6th segment. b. Horizontal section of the ganglion of the 6th segment. The level at which the horizontal section was made is shown by bars at the margins of (a). L: immunoreactive large cells, NL: non-immunoreactive large cells, NP: neuropile, NS: non-immunoreactive small cells, S: immunoreactive small cells, SN: segmental nerve. Scale, 50 μm.

bilaterally and extended as far as the body wall (Fig. 8a). Thus, three immunoreactive fiber tracts were clearly seen between the circular and longitudinal muscle layers of the body wall (Fig. 8b). The immunoreactive fibers in these tracts seem to branch off after they reach the body wall. Thus, there were many immunoreactive deposits indicating the immunoreactive fibers, between the circular muscle layer and the epidermal cell layer of the skin (Fig. 8b). The immunoreactive fibers were also found in the circular and longitudinal muscle layers (Fig. 8b). FMRFamide-like immunoreactive cells were occasionally found between the epidermal cells of the skin (Fig. 8c).

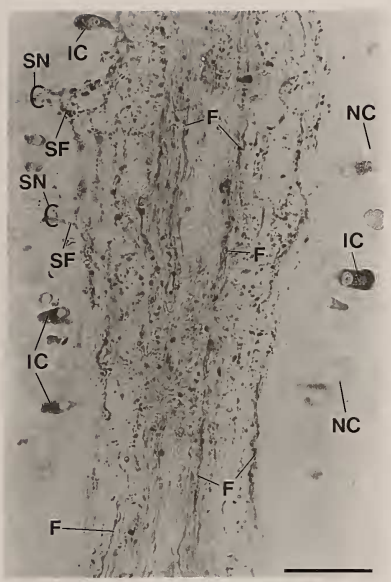

FIG. 7. Horizontal section of the ventral nerve cord of the segment in the latter half of the body, showing immunoreactive cells (IC) and two wide nerve tracts containing FMRFamide-like immunoreactive fibers (F). The section was made at the level of the neuropile shown in Fig. 6a. NC: non-immunoreactive cells, SF: immunoreactive fibers proceeding along segmental nerve (SN). Scale, 50 μm.

Alimentary canal

Immunoreactive nerve fibers were observed between the epithelial cell layer and the muscle layer of the pharynx (Fig. 9a), esophagus (Fig. 9b) and intestine (Fig. 9c). However, the numbers of immunoreactive deposits, which indicate the presence of immunoreactive fibers, were smaller in the anterior portion than the posterior portion of the pharynx (cf. Fig. 2a, b). Immunoreactive cells were occasionally found between the epithelial cells of the alimentary canal (Fig. 9b).

DISCUSSION

The existence of immunoreactive FMRFamide-like peptides in the central nervous system has been studied phylogenetically in many animal species (see Introduction for references). In the case of annelids, however, there is only one relevant report and it describes the presence of FMRFamide-like immunoreactive cells in the brain and segmental ganglia of the medicinal leech [29]. The present study has revealed the dense distribution of FMRFamide-like immunoreactive nerve cells and fibers in the cerebral, subesophageal and segmental ganglia, and in the ganglia of the pharyngeal plexus in *Eisenia foetida*. These findings indicate that the earthworm possesses immunoreactive FMRFamide-nervous system, which is similar to the immunoreactive serotonin-nervous system [7, 8]. In addition, the presence of immunoreactive FMRFamide in the earthworm is considered to afford further evidence that FMRFamide-related peptides have had a long evolutionary history, since they are found in vertebrate nervous system [25].

In *E. foetida*, the immunoreactive nerve bundles proceeding from the cerebral ganglion towards the prostomium probably innervate peripheral organs of the anterior part of the body and release FMRFamide or related peptides as neurotransmitters or neuromodulators. The immunoreactive fibers proceeding ventro-posteriorly from the cerebral ganglion along the pharyngeal connectives may transfer information to the subesophageal ganglion, and further to the segmental ganglia directly or through the subesophageal ganglion. In the cerebral ganglion, there were cell groups consisting of small or large immunoreactive cells and they were located in different regions. At the present time, it is not known which cell types and groups extend their nerve fibers to the prostomium or the subesophageal ganglion.

The subesophageal ganglionic cells extend immunoreactive fibers towards the ventral region of the anterior segments. Further, the subesophageal ganglion projects immunoreactive fibers posteriorly towards the segmental ganglia. Thus, the immunoreactive fibers connect the subesophageal ganglion with the cerebral and segmental ganglia.

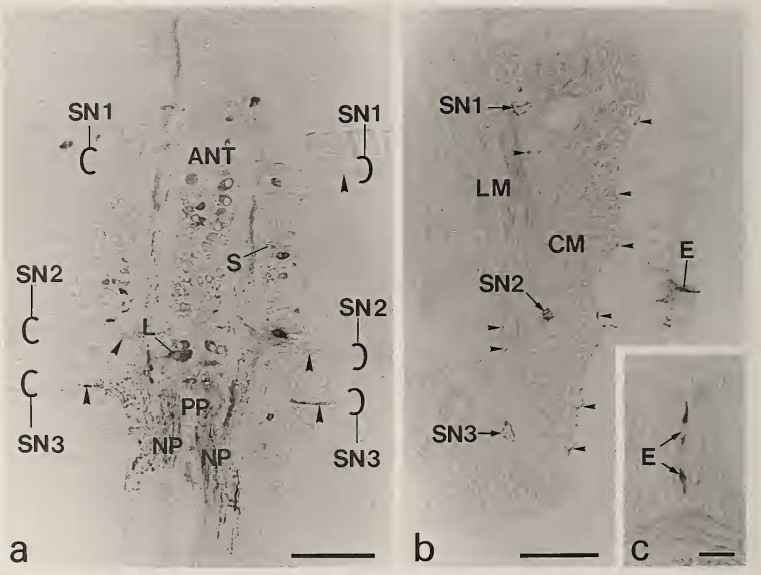

Fig. 8. a. Horizontal section of the segement in the latter half of the body. Three segmental nerve trunks (SN1, SN2, SN3) including immunoreactive fibers (arrow heads) extend to the body wall on both sides. The anterior part (ANT) is a cut at the same level as in Fig. 6b and the posterior part (PP) is a cut at the same level as in Fig. 7. b. Horizontal section of the skin of the anterior 8th segment, showing three segmental nerve tracts (SN1, SN2, SN3) including immunoreactive fibers. They were located between the circular (CM) and longitudinal (LM) muscle layers of the skin. c. Immunoreactive cells (E) between the epidermal cells of the skin near the rostral end of the body. L: immunoreactive large cells, NP: neuropile, S: immunoreactive small cells. Arrow heads: immunoreactive deposits. Scale, a: 100 μm, b: 100 μm, c: 20 μm.

It is possible that information transfer may take place from the segmental ganglia to the subesophageal and cerebral ganglia through the immunoreactive FMRFamide fibers.

The immunoreactive fibers in the segmental nerve trunks proceeding to the body wall seem to innervate the epidermal cells and the circular and longitudinal muscles, as suggested earlier by electrophysiological studies [30]. It seems that immunoreactive FMRFamide fibers may be involved in the regulation of activities of the epidermal cells and the integumental muscles. Further, the immunoreactive cells found between the epidermal cells of the skin are most probably sensory cells.

The fibers immunoreactive to FMRFamide antiserum in the pharynx must be derived from the ganglia of the pharyngeal plexus, since it has already been shown by anatomical and histological techniques that the pharynx is innervated by neurons in the ganglia of the pharyngeal plexus [30, 31].

The immunoreactive fiber connection between the ganglia of the pharyngeal plexus and the pharyngeal connectives may contribute to information exchange between them. Nervous connections between them have already been demonstrated by a silver nitrate impregnation technique [31]. The esophagus and the intestine seem to be

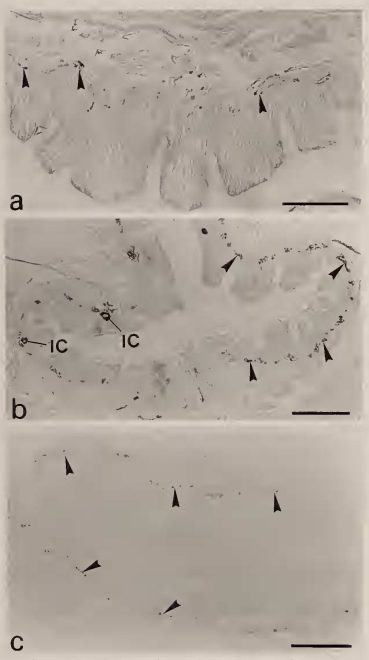

FIG. 9. Horizontal sections of the pharynx (a), esophagus (b) and intestine (c) showing FMRFamide-like immunoreactive nerve fibers (arrow heads) between the epithelial layer and the circular muscle layer. IC: immunoreactive cells. Scale, a: 50 μm, b: 100 μm. c: 100 μm.

innervated by the immunoreactive fibers that originate in the subesophageal ganglion and the segmental ganglia, respectively. It has been shown electrophysiologically that nerve fibers proceed to those organs via the body wall and the septa [30].

Considering all the findings mentioned above, it is clear that immunoreactive FMRFamide-like peptides function as neurotransmitters or neuromodulators. However, they may also have neurohormonal functions, since we found immunoreactive products in close contact with the blood vessels in the cerebral and subesophageal ganglia of *E. foetida*. Thus, our findings suggest that they can serve as neurotransmitters or neuromodulators in some cases and neurohormones in other cases, as discussed by other investigators [18, 21–27, 32–38].

In the present study, the immunoreactive nerve fibers originating from the segmental nerve trunks were found between the epithelial cell layer and the circular muscle layer of the alimentary canal. These findings suggest that immunoreactive FMRFamide is involved in the regulation of the activity of the epithelial cells of the alimentary canal.

It has been demonstrated that FMRFamide-like peptides are involved in cardiovascular control in molluscs [18, 38]. In the annelids, Kuhlman *et al.* [35] suggested that FMRFamide-like substances function in chemical transmission within the heartbeat system of the leech. Although we could not detect FMRFamide-like immunoreactive fibers in the heart of the earthworm, immunoreactive FMRFamide may affect the heart as a neurohormone after it has been released into the blood vessels.

As mentioned in the introduction, many neuropeptides, such as PP, VIP, α-endorphin, β-endorphin, ACTH, SP, Leu-enkephalin, Met-enkephalin, gastrin/CCK, CRF, h-GHRF, dynorphin, OXT and AVP, have been demonstrated immunohistochemically in the nervous system of the earthworm. We have added one more neuropeptide, FMRFamide-like substance, to those peptides found in the earthworm's nervous system. In the present study, we found many neurons non-immunoreactive to FMRFamide antiserum in all the ganglia. These neurons may contain serotonin, catecholamines or other peptides than immunoreactive FMRFamide. Physiological functions of these peptides are not clear at the present time.

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